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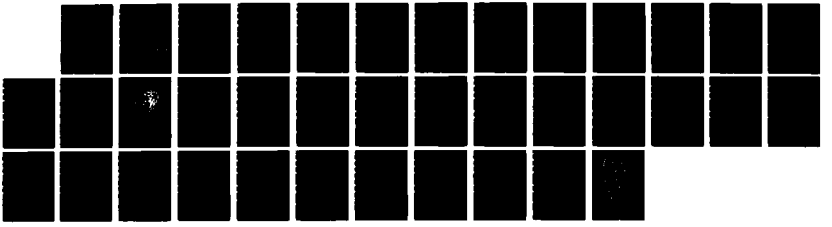

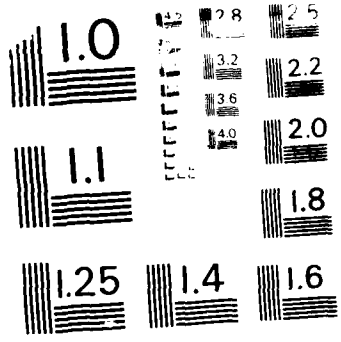

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Chemical Blistering: Cellular and Macromolecular Components

Annual Report

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The overall objective of this investigation is to elucidate the molecular mechanisms by which HD exerts its chemical blistering action when applied topically to human skin. The study involves the use of keratinocyte tissue cultures in order to focus on the direct interactions between the mustard and the cellular targets. The technical objectives of the project are to develop appropriate culture systems for use in the investigation of subcellular and macromolecular manifestations of toxicity, to establish the credibility of these systems for investigating the molecular mechanisms of these effects and to determine whether these systems can be used to develop procedures by which toxic responses can be neutralized.

The following three biological systems have been developed with which to carry out the study: a stratified terminally differentiating system, grown at air/liquid interface on a collagen substratum or a nylon membrane substratum with which to study the effects of HD on epidermal differentiation, a submerged monolayer of proliferating

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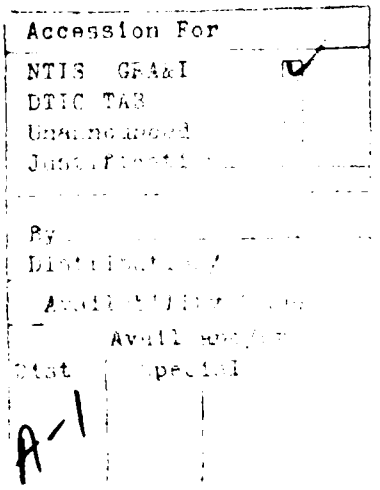
keratinocytes which can be used to elucidate the molecular and cellular effects of the toxicant on epidermal proliferation, and purified populations of basal and differentiated (mixed) cells which can be compared for sensitivity to the toxic effects of the mustard.

The experimental rationale of the project is to define those parameters of toxicity which appear in the cultures at the lowest exposures to HD, e.g. alkylation of DNA, inhibition of DNA repair, and then determine the relevance of these effects to the cellular human skin in vivo. It has been determined that the effects of DNA occur at levels of exposure below those needed to cause an abnormality in the respiration of mitochondria, glycolysis, utilization of glucose, protein synthesis or RNA synthesis. It is possible, however, that effects on metabolic and ultrastructural parameters are necessary to obtain the cellular necrosis. The further effort of this investigation is designed to determine dose response curves for these and other parameters of toxicity in order to define the minimal profile of effects which are necessary in order to observe the cellular degeneration which is thought to be the progenitor of vesication in human skin.



I. SUMMARY

The overall objective of this investigation is to elucidate the molecular mechanisms by which bis-(beta-chloroethyl)sulfide (BCES) exerts its vesicant action when applied topically to human skin. The study will be done using cultures of cutaneous keratinocytes in order to focus on the direct interactions between the mustard and the cellular targets.

The technical objectives of the project are to develop appropriate culture systems for use in the investigation of subcellular and macromolecular toxic manifestations, to establish the credibility of these systems for investigating the molecular mechanisms of these effects and to determine whether these systems can be used to develop procedures by which toxic responses can be neutralized.

During the first two years of this project, protocols were established for growing human and rat keratinocytes on collagen gel and nylon membrane substrata at the air/liquid interface in order to produce an "epidermis" *in vitro* which by morphological and biochemical criteria was similar to the epidermis *in situ*. The morphological criteria were shown to be satisfactor during the second year of the project. During the current year, biochemical criteria were investigated and also shown to be satisfactory. Lectin-binding studies have demonstrated that as in the tissue *in situ*, the cells in the culture change their carbohydrate structures on the cell surface. The culture also exhibits the same maturation of keratin peptides as is seen in the intact tissue. This observation was made with monoclonal keratin antibodies having separate specificity for early and late keratins. In addition, a monoclonal antibody for filaggrin was shown to localize in the granular cells when applied to a lifted culture.As yet, these biochemical parameters have only been determined on cultures grown on collagen.

A second type of culture available for this study is a submerged monolayer of proliferating and early differentiated keratinocytes. This culture has to date been grown on the plastic surface of the Petri dish. Stratification in this culture is inhibited by using a low level of calcium ion (0.08 - 0.10 uM) in the medium. Although this culture was reported in the literature to be composed of proliferating cells, lectin-binding studies have now revealed that there are a considerable number of early spinous cells in the monolayer. This culture can be used to identify target molecules for BCES since the toxicant is always in direct contact with the target cells in this biological system. This type of culture can be obtained from human as well as animal cells.

In developing the techniques for obtaining appropriate innocula for cell cultivation, procedures became available by which purified populations of basal cells can be obtained. These populations are about 98% pure in terms of morphology but, again, lectin-binding studies have indicated that the preparations have small percentages of cells which have the carbohydrate surface structure of spinous cells. Nevertheless, these preparations are sufficiently pure to be useful in studying the effect of BCES on the differentiative capabilities of keratinocytes. In the process of obtaining populations of basal cells, populations of the various differentiated types

can be obtained as a mixture. Centrifugation in a self forming density gradient of Percoll is used to obtain these populations. To date, this preparation has only been obtained from animal cells. There seems no reason to doubt that the procedure can be applied to human cells as well.

Application of BCES to the lifted cultures can be done in 70% ethanol or dimethylsulfoxide, the latter being preferred. The solvent itself has some effect on the culture as observed biochemically but there seems to be no convenient way around this problem given the solubility and stability characteristics of BCES.

Preliminary work has been accomplished directed at identifying the most sensitive parameter of toxicity from exposure of the culture to BCES. Lower exposures affected the integrity and metabolism of DNA than the utilization of glucose, glycolysis, protein synthesis, RNA synthesis and the respiration of mitochondria. At an exposure level of 5 - 10 uM BCES, DNA is still damaged but the cells have some capability of repairing their DNA.

Glutathione S-transferase and peroxidase, two enzymes which might influence the toxicity of BCES by inactivating the toxicant, were previously identified as present in the epidermis and have now been purified from whole skin.

The biological systems developed in this project are appropriate for use in determining the mechanisms responsible for the molecular and cellular manifestations of toxicity from BCES. Attention should now be focused on determining the most sensitive toxic responses and identifying those responses which are primarily responsible for those manifestations of toxicity which are requisite for vesication.

II. FOREWORD

The source of animal tissue for primary cultures described in this report was neonatal rats derived from the CFN strain by random mating reared in the School of Public Health's animal facility. This facility is under the supervision of the University Unit for Laboratory Animal Medicine. The University of Michigan is accredited by the American Accreditation Association for Laboratory Animal Care (AAALAC). In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals" prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research council [DHEW Publication No. (NIH) 78-23, Revised 1978].

The source of human tissue was foreskin obtained at routine circumcisions done at Women's Hospital, The University of Michigan, and provided without identification of the donor. The form utilized to obtain "informed consent" was the one in use by the hospital for routine circumcision. Signature of this form allows experimental use of tissues. The use of this tissue for purposes of the present project has received approval by a University Human Subjects Review Committee and, for the protection of human subjects, the investigators have adhered to policies of applicable Federal Law 45 CFR 46.

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III. NARRATIVE REPORT OF PROJECT PROGRESS

A. Statement of the Problem under study

The overall objective of this investigation is to elucidate the molecular mechanism by which bis-(beta-chloroethyl)sulfide (BCES) exerts its vesicant action when applied topically to human skin. The theoretical and technical advances which have occurred in cutaneous biology over the past several decades encourages the view that this objective can be achieved. It appears likely that the most productive application of the new knowledge would be in experiments involving cultures of epidermal keratinocytes. Studies *in vitro* should allow better controlled experiments and yield more reproducible data than is true *in vivo*.

The technical objectives of this project are to develop appropriate culture systems of epidermal keratinocytes for use in the study of subcellular and macromolecular toxic manifestations resulting from environmental exposure to irritant chemicals - particularly BCES, to establish the credibility of these systems for investigating the molecular mechanisms of these effects and to determine whether these systems can be used to develop procedures by which toxic responses can be neutralized.

The *specific aims* which guide the study are as follows:

1. To develop *in vitro* systems (i.e. purified populations of basal and differentiated cells) which can be used to elucidate the molecular mechanisms responsible for damage in the mammalian cutaneous epidermis exposed to BCES;

2. To identify changes in morphological and/or biochemical parameters (e.g. ultrastructure, structure and metabolism of DNA, enzymatic activity and physiological function) which can be used *in vitro* as early indicators of the type of chemical destruction that is associated with intraepidermal vesication such as occurs from exposure to BCES;

3. To understand the molecular mechanisms by which topically applied BCES causes the initial preferential destruction of the epidermal basal and lower spinous cells, and

4. To identify techniques for arresting, reversing and/or neutralizing the biochemical, ultrastructural and cytochemical effects in epidermal cells exposed to BCES.

B. Literature Background

1. Anatomy of blistering

Application of BCES to human skin results in an initial erythema followed by blistering. Stoughton (1971) has noted that in vesication "fluid accumulation is almost always secondary to fundamental damage to the cellular structures" and has defined a blister as an abnormal accumulation of fluid, completely replacing the pre-existing tissue structure, capped by

a part or all of the epidermis." The blister seen after exposure to BCES fits this description. The progression to ultimate blistering from this agent proceed irrevocably unless the action of the chemical is neutralized within the first several minutes of exposure. Warthin and Weller (1919) and Sinclair (1949) noted that the process initially involves destruction of the basal and lower spinous layers of the epidermis. Presumably, the cellular destruction that produces a cavity in the tissue precedes the actual fluid accumulation. At later stages in the destructive process, the necrosis may spread to the upper spinous and granular layers as well as into the dermis. Blistering is rare in animals and appears to be a reaction primarily seens in man in whom it may arise as a component of various cutaneous diseases and as a result of exposure to some biological (e.g. Herpes virus), physical (e.g. ultraviolet radiation) or chemical (e.g. mustards) stressors. The blister may develop at different levels in the skin depending upon the etiologic agent involved.

2. Biochemistry of blistering

Since different vesicants produce blisters at different levels in the tissue, it would not be unexpected if the biochemistry involved were different depending on the location of the blister.

Exposure to a mustardin vivo, will cause separation at the dermal -epidermal junction i.e. the blister appears at that level in the tissue. From a description of the time course of dermatopathologic developmentd and the time postexposure within which the process can be reversed, it is clear that the molecular course of the pathologic process is set within the first 3 min of exposure in vivo. Warthin and Weller (1919) found that erytheme, inflammation and vesication were not reversed by therapy (i.e. application of chlorinated lime) initiated after 3 min of exposure to BCES.

The molecular mechanism of BCES's effect is unknown but substances in this class are powerful alkylating agents of DNA and alkylated DNA could lead to inhibition (or at least delay) of replication, to generalized breakdown of damaged DNA leading to cell death (cf Wheeler, 1962) and to low-fidelity repair resulting in mutations with consequent disruption of normal metabolic function (Kirner, 1946, Wheeler, 1962) Mustards can also alkylate RNA with consequent interference in the translation of genetic information and protein (Ross, 1962) resulting in metabolic disruption (Wheeler, 1962). BCES, being a bifunctional mustard can also cross-link DNA and DNA to RNA or to protein. The most important molecular target appears to be the DNA (Fox and Scott, 1980). BCES alkylates and cross-links at the purine bases. Alkylation of the phosphate groups in DNA can also occur. On the basis that damaged proteins can be replaced whereas damaged DNA may be irrevocably harmed, the DNA would seem to be the most important target of toxicity by BCES.

Exposure of isolated skin to a vesicant for as little as 5 min, can result in the inhibition og glycolysis and respiration (Barron, et al., 1948). Glycolysis is inhibited by a lower concentration of toxicant that is necessary to obtain inhibition of respiration. In the case of exposure tomustards, this effect seems to be a consequence of a reduced level of

pyridine nucleotides in the cell (Holzer and Kroger, 1958; Frazer, 1960). Recent evidence suggests that BCES causes a lowered level of pyridine nucleotides by virtue of the stimulation of polyADP polymerase ross, et al., 1983).

Glutathione affects the toxicity of BCES because most of the BCES that enters the body is conjugated with glutathione and excreted (Davison, et al., 1961). Minor urinary excretory products may include bis-cysteinyl-ethyl sulfone and thiodiglycol formed non-enzymatically (Roberts and Warwick, 1963).

3. Repair of alkylated DNA

In bacteria, the ability to excise BCES-alkylated products from DNA is associated with increased resistance to the mustard (Lawley and Brookes, 1968). Apparently both mono- and bifunctional adducts can be removed from DNA since comparable amounts of induced repair synthesis have been observed in HeLa cells exposed to doses of BCES and the analogous half-mustard, both compounds being equally toxic (Roberts, et al, 1971). However, there appears to be disagreement as to the relative rates at which repair of the two types of lesion occur (Reid and Walker, 1969; Roberts, et al, 1971). Given the mechanisms probably involved in the two repair processes,it seems unlikely that removal of the cross-links could occur as quickly as repair of monofunctional adducts (cf Fox and Scott, 1980). Intuitively, it appears more likely that that the cross-link would be removed one arm at a time rather than both arms simultaneously since the latter process would insert a double strand break which could be lethal. Of course, this assumes that the mechanism of repair is not simple base replacement. Detailed information on the molecular mechanisms by which BCES-induced lesions in DNA are repaired does not seem to be available.

4. Cultivation of keratinocytes

The two main types of cells in the skin are fibroblasts (dermal) and keratinocytes (epidermal). Only the keratinocytes existing at the dermal - epidermal junction can double their DNA and undergo mitosis although all nucleated keratinocytes can repair their DNA (Karasek and Moore, 1970; Vaughan and Bernstein, 1971). Fibroblasts are easily cultivated using basal medium supplemented with serum (Earle, 1958). Cultivation of keratinocytes requires more stringent attention. Several systems are available for cultivating keratinocytes. The best involve use of a substratum of collagen (Karasak and Charleton, 1971; Freeman, et al., 1976) or a feeder layer of irradiated fibroblasts (Rheinwald and Green, 1975). Conditioned medium is also useful (Ham, 1982) but not necessary (Peehl and Ham, 1980; Eisinger, et al, 1980).Good growth can be obtained by supplementing the medium with growth factors such as epidermal growth factor (Cohen and Savage, 1974); Rheinwald and Green, 1977) and hormones (Hayashi, et al, 1978). Vaughan, et al.(1981) reported successful cultivation, passage and increased plating efficiency of murine keratinocytes after supplementing basal medium with hydrocortisone and insulin.

Most of the cultures of keratinocytes alluded to above, form

monolayers with some multilayering and production of cornified layers. However, they do not reproduce structural characteristics typical of their counterparts *in situ*. There have been previous reports of stratification of rabbit, human and rat keratinocytes with some cellular characteristics similar to intact epidermis (Karasek and Moore, 1970; Vaughan and Bernstein, 1971; Kitano, 1979). Lillie, at al. (1980) cultured rat lingual epithelial cells at the air/liquid interface by lifting collagen-supported cultures on organ culture grids. This resulted in stratification and terminal differentiation with organellar components similar to the parent tissue. Modified application of this technique to primary isolates of cutaneous keratinocytes from the skin of newborn rats and from human infant foreskin in this laboratory, has produced an "epidermis" which by morphological criteria is similar to that of the tissue *in situ* (Bernstein, et al.,Annual Report, DAMD17-82-C-2196, University of Michigan to USAMRDC, November 15, 1984). These morphologic criteria include the presence of desmosomes, bundles of intermediate filaments (i.e. tonofilaments), keratohyaline-like granules and an extensive orthokeratotic cornified layer.

C. Experimental Rationale for the Investigation

A tissue culture of cutaneous keratinocytes provides a biological system in which the direct interaction of BCES with molecular and cellular elements can be studied without the systemic influences which secondarily affect the toxic manifestations. If the culture were to have the morphological and biochemical characteristics of the epidermis *in situ*, the mode of human exposure, topical application, could be mimiced experimentally and the results would probably have more credibility in terms of application to the situation, *in vivo*. Furthermore, if the culture were to use human keratinocytes, the study would be as close to the "human condition" as one could get without trauma to a human volunteer. Therefore, the main system to be developed in this investigation was to be the stratified terminally differentiating culture of human cutaneous keratinocytes. En route, several other systems would be used including a proliferating monolayer of keratinocytes and cultures of keratinocytes derived from the skin of the newborn rat. Additionally, an effort would be made to isolate and purify populations of basal and differentiated cells respectively.

Molecular parameters of toxicity were to be tested in the monolayer submerged monolayer culture to establish a range of effective exposures to BCES. The intent was to determine which indicators of toxicity appeared at the lowest exposure and then to determine whether these parameters are indeed relevant to toxicity as observed in stratified cultures after topical application of BCES at dosages equivalent to those which produce a toxic response *in vivo*.

D. Progress Report (15 September 1984 - 14 September 1985)

The specific aims of the original contract proposal and progress toward their achievement during the above noted period are as follows:

1. To develop in vitro systems which can be used to elucidate the molecular mechanisms responsible for damage in the mammalian cutaneous epidermis exposed to BCES.

a. Development of protocols for obtaining stratified, differentiated cultures of keratinocytes

1). Determination of the effect of added factors on attachment and proliferation of keratinocytes on various substrata.

Several factors are employed in cell cultivation to enhance the attachment and subsequent growth of mammalian cells in vitro. Attachment factors that in vivo seem to play a role in the development of epithelial tissues, have been investigated to enhance attachment and proliferation. Such factors are found in the basement membranes of these tissues and two of them, i.e., laminin and type IV collagen have been isolated in relatively pure form. Several experiments have been conducted using these factors, alone or in combination, to precoat plastic and synthetic membranes as substrata. These factors have also been compared with another biologically active factor, human fibronectin (Collaborative Research Laboratories), which is widely used in cell culture.

Laminin (Bethesda Research Laboratories) supported attachment of keratinocytes to both plastics and membranes much better than did human fibronectin. In fact, keratinocytes seeded on surfaces coated with the latter did not attach as well as they did on plastics while those seeded on laminin coated surfaces attached better. For example, 20% more cells attached to TCM-200 filters coated with laminin than to untreated ones although monolayers of equal confluency usually resulted in both after incubation for 7-10 days. This was determined by counting stained cells in a given growth area. However, a very serious problem was discovered concerning the precoating of surfaces with laminin for keratinocyte cultivation. After approximately 14 days, the culture began to detach from the surface of the substrate as a complete sheet and the culture was thus lost. We have no explanation for this phenomenon at this time but it prevents the use of this attachment factor if long-term cultures are needed for experiments.

Type IV collagen (Bethesda Research Laboratories) is being studied. There are several possible biological sources of Type IV. The one that has been studied so far, is proving to be unsatisfactory. It cannot be made into a gel but must be air dried on a surface. No increase has been observed in cell attachment using this material in this manner. None of the attachment factors used to precoat membranes, increased attachment and proliferation of rat keratinocytes over that seen with untreated membranes. Untreated nulon membranes obtained from Gelman produced results

superior to all other membranes and plastics treated or untreated.

b. Submerged low-calcium cultures of murine keratinoctes

Cultured keratinocytes can be kept as a monolayer by restricting the level of Ca^{2+} in the medium (Hennings, et al.,1980). Although stratification is retarded under these conditions, differentiation does occur. As noted below, the differentiated cells seem to be primarily at the lower spinous stage of the keratinization process.

To date, monolayer cultures have been grown on plastic surfaces and not on collagen or other substrata. Populations of cells obtained by trypsinization of skin were seeded into culture dishes and allowed to grow in medium consisting of calcium-free MEM supplemented with 10% Chelex-treated fetal calf serum and then adjusted to 0.11 mM Ca^{2+} with calcium chloride. Such monolayer cultures exhibited the typical cobblestone pattern of distinct cells with large intercellular spaces (Figure 1). This culture can be maintained for more than 6 weeks and consists of both mitotically-capable cells and cells which appear to be unable to divide. These may be differentiated cells since some of the cells bind the Ulex europeus lectin. In tissue sections of skin, this lectin binds to the epidermal spinous cells (cf Brabec, et al., 1980). The methodology of cultivation for this preparation were worked out in context of another ongoing project of this laboratory. A description of the technique and the morphology of the culture has been published (Brown, et al., 1985).

c. Comparison of cytochemical parameters between epidermis, in situ, and cultures of keratinocytes.

Monolayer cultures of rat keratinocytes grown in reduced Ca^{2+}, exhibit lectin binding on the cell surface which is consistent with the picture, in situ. Brabec, et al. (1980) demonstrated the binding of Bandeiraea simplicifolia (now called Griffonia simplicifolia), Isolectin I-B_4, to the surface of basal cell and the cells of the lower two spinous layers. On the other hand, Ulex europeus Agglutinin I (UEA) was seen to bind to the surface of the spinous cells and not to the basal cell. Each lectin binds a surface receptor having a particular carbohydrate as the non-reducing terminal of the receptor glycoconjugate, e.g. the I-B_4 to methyl-α-D-galactoside and [illegible] to α-L-fucose. In the developing monolayer culture, early during the period of cultivation, the cells bound primarily I-B_4. Later as they differentiated, the cells also bound the UEA. These observations in vitro would be expected from the data obtained by Brabec et al (1980) in vivo.

Lifted human cultures exhibited antibody staining that also was consistent with data obtained in vivo. In cultures grown submerged for ? weeks and lifted for 3 weeks, a monoclonal anti-keratin (2D6) which immunolocalizes to only basal cells in tissue sections of human skin, bound only to cells in the basal layer of the culture. Also AE2 antikeratin (Sun, 1983) which localizes over granular cells in situ, labeled cells under the cornified layer in culture.

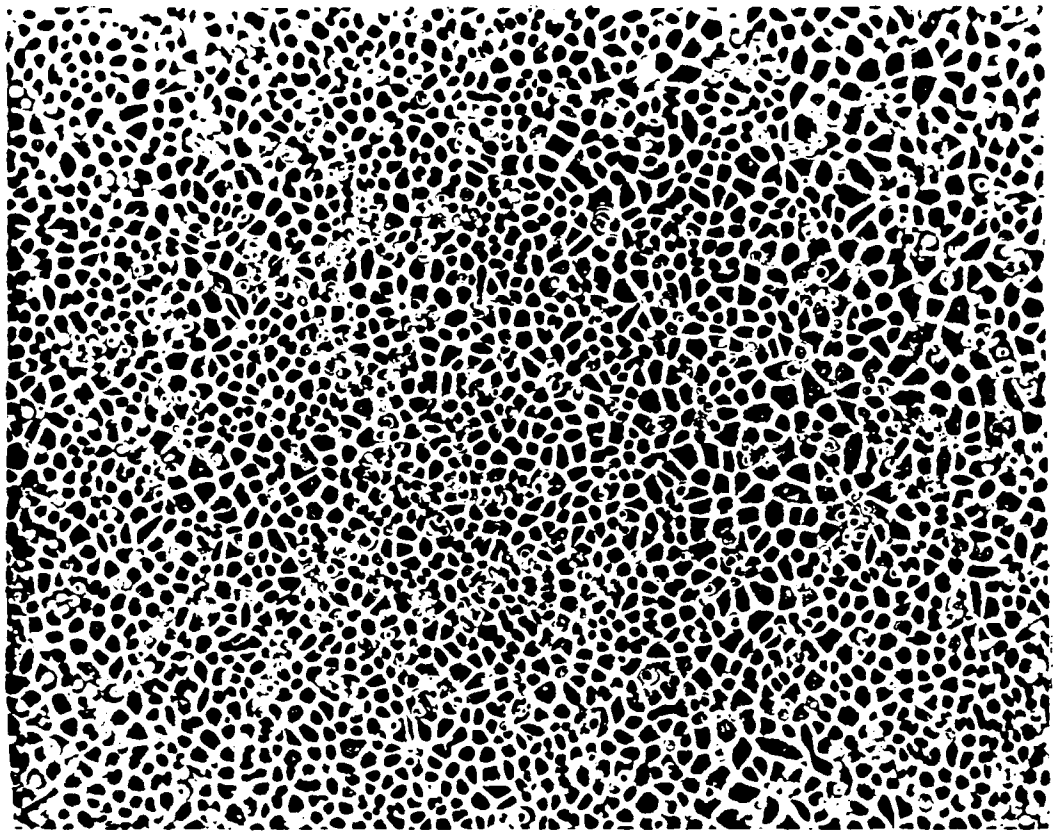


Fig. 1 Phase contrast micrograph of rat keratinocytes, grown for 7 days in 0.1 mM Ca^{++} with 10% Chelex treated FCS. 225x

2. To understand the molecular mechanisms by which topically applied BCES causes the initial preferential destruction of the epidermal basal and lower spinous cells.

a. Choice of solvent for topical application of BCES. Studies of solvent effects on the culture.

Because of the ease of preparing nylon membranes for keratinocyte cultivation, this membrane has been used to study the effect of various solvents on culture viability. The proper, non-toxic solvent for BCES that will result in uniform distribution of the agent over the surface of the culture is being sought. Acetone, ethanol, hexane and methylene chloride as nonaqueous and dioxane and dimethylsulfoxide as aqueous solvents, are being considered.

Acetone, hexane, methylene chloride and absolute alcohol were found to be quite toxic to the differentiated cultures when applied topically. Evaporation was immediate and the consequent drastic drop in temperature may have caused the irreversible cell damage which was observed as a decrease in the incorporation of DNA and protein precursors. Various percentages of alcohol were also evaluated for cytotoxicity. The highest concentration of ethanol which was tolerable was found to be 40%. This concentration produced only mild toxicity from which the cells recovered. However, it was found that BCES in methylene chloride does not dissolve in 40% ethanol to the extent desirable. On the other hand, dimethyl sulfoxide (DMSO) was found to be satisfactory. A comparison of the effects of the latter two solvents on the incorporation of labeled thymidine and leucine is shown in Table 1. The data demonstrate that exposure of differentiated cells to 70% DMSO affected metabolic activity to an extent that was similar to the effect after exposure to 40% ethanol.

DMSO was shown to be the least toxic of the effective solvents for BCES thus far examined. Experiments have been initiated to determine the effect of the solvent and various concentrations of BCES on the capacity of differentiated cultures to incorporate radioactive precursors of DNA and protein. In these experiments, lifted cultures grown for 14 days were exposed to 50, 100 and 200 nM BCES dissolved in 70% DMSO for 1 and 2 hr, pulse labeled with a labeled precursor for 0.5 hr and then prepared for liquid scintillation counting of incorporated tracer. Figures 2 and 3 show the incorporation of thymidine and leucine, respectively, into acid precipitable macromolecules in relation to the time of exposure to BCES. As shown in the figures, there was an initial inhibition of incorporation which increased as the dosage increased. After the initial inhibition of incorporation, there was a stimulation which exceeded the control level as incubation continued. It appears that the damage occurs early after exposure to BCES and experiments are now under way to measure this effect earlier than 30 min after exposure.

TABLE 1

Incorporation of ^{3}H-thymidine and ^{14}C-Leucine by Lifted Cultures After Exposure to Solvents.

Treatment	Thymidine Exposure time (Hrs) 2.5	Thymidine Exposure time (Hrs) 24	Leucine Exposure time (Hrs) 2.5	Leucine Exposure time (Hrs) 24
Untreated control	1185 ± 279	798 ± 125	2.69 ± 0.6	1.87 ± 0.19
70% DMSO	658 ± 138	564 ± 110	1.27 ± 0.03	1.42 ± 0.29
40% ETOH	836 ± 82	542 ± 162	2.05 ± 0.78	1.12 ± 0.24
Heat killed control		83 ± 21		0.2 ± 0.06

b. Development of an assay to measure fidelity in DNA repair

The use of viruses to investigate DNA repair has become widespread (Defais, et al.,1983). In most such investigations, some modification of the technique of "host cell reactivation," has been applied. In principle, this experimental technique involves infection of a permissive host with a virus which is non-virulent because of damaged DNA. The method depends on cellular repair processes to reverse the damage and reactivate the virus to virulence. This technique is to be used to determine the efficacy of the host cell's repair system and to evaluate the possible effects of a toxic chemical on the repair process. By inserting a specific lesion into the viral DNA, it should be possible to evaluate the repair system for that particular type of lesion. This technique is being developed for use in evaluating the effect of exposure to BCES on the "error free" repair capability of exposed keratinocytes.

Specifically, the probe will be a heteroduplex of SV 40 DNA containing two mismatched base pairs which confer temperature sensitivity on the large T antigen genome. This DNA will be used to transfect a culture of human keratinocytes which is semipermissive for SV 40. Repair will then be allowed to occur at a non-permissive temperature so that only the repaired DNA, i.e. the non-temperature sensitive genome, will be transcribed and translated to form the large T antigen. T antigen must be made in order for the viral DNA to be replicated. When T antigen is made from temperature sensitive genome, the protein is not stable and viral DNA is not replicated. Repair of this damaged DNA will be observed by noting the time it takes for the host cells to repair the DNA and the percentage of transfected cells which carry out the repair. The end point assay will be the appearance of new viral DNA (determined by gel electrophoresis) or the appearance of viral plaques.

Figure 2

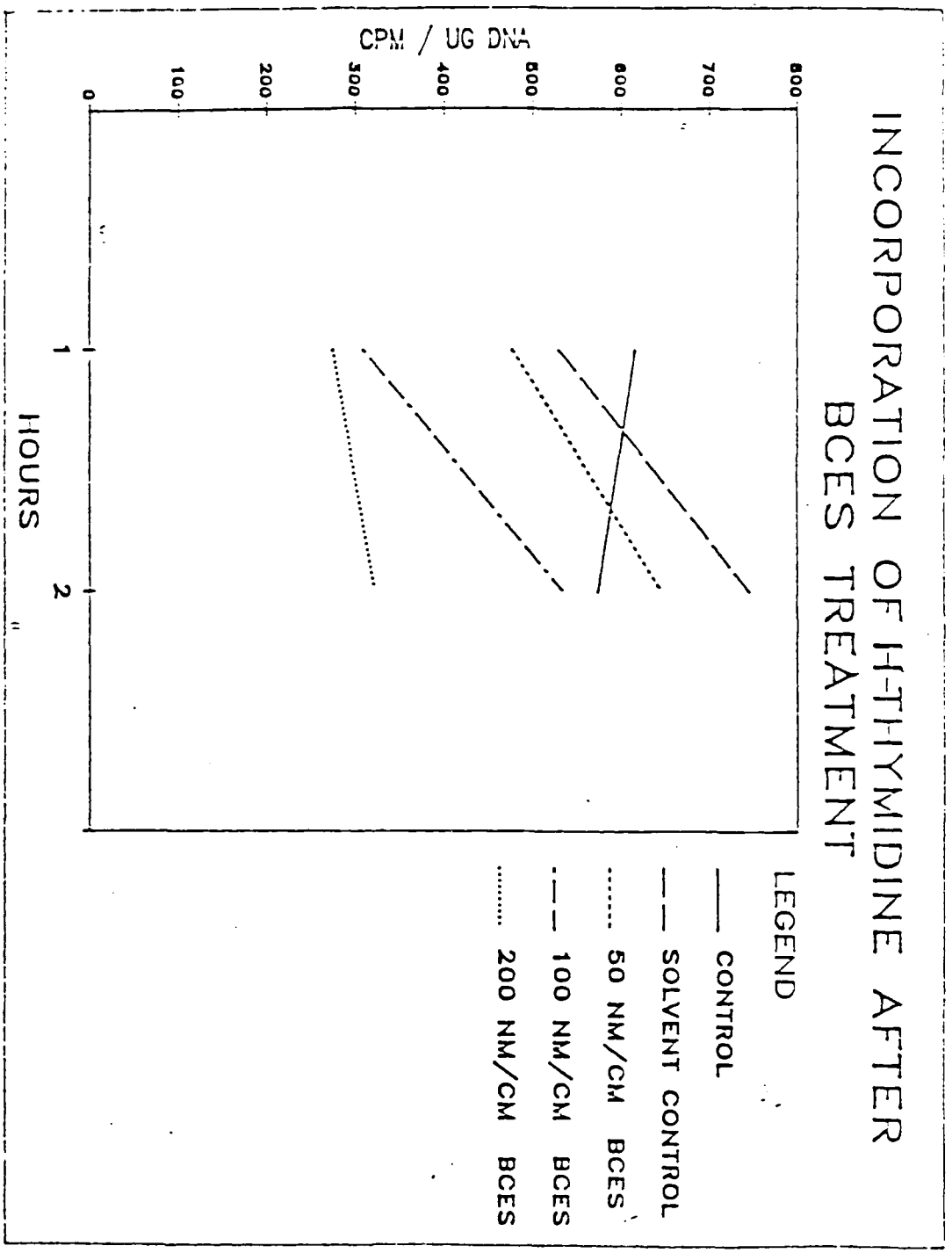

INCORPORATION OF H-THYMIDINE AFTER
BCES TREATMENT
CPM / UG DNA
0
100
200
300
400
500
600
700
800
1
2
HOURS
LEGEND
CONTROL
SOLVENT CONTROL
50 NM/CM BCES
100 NM/CM BCES
200 NM/CM BCES

Figure 3

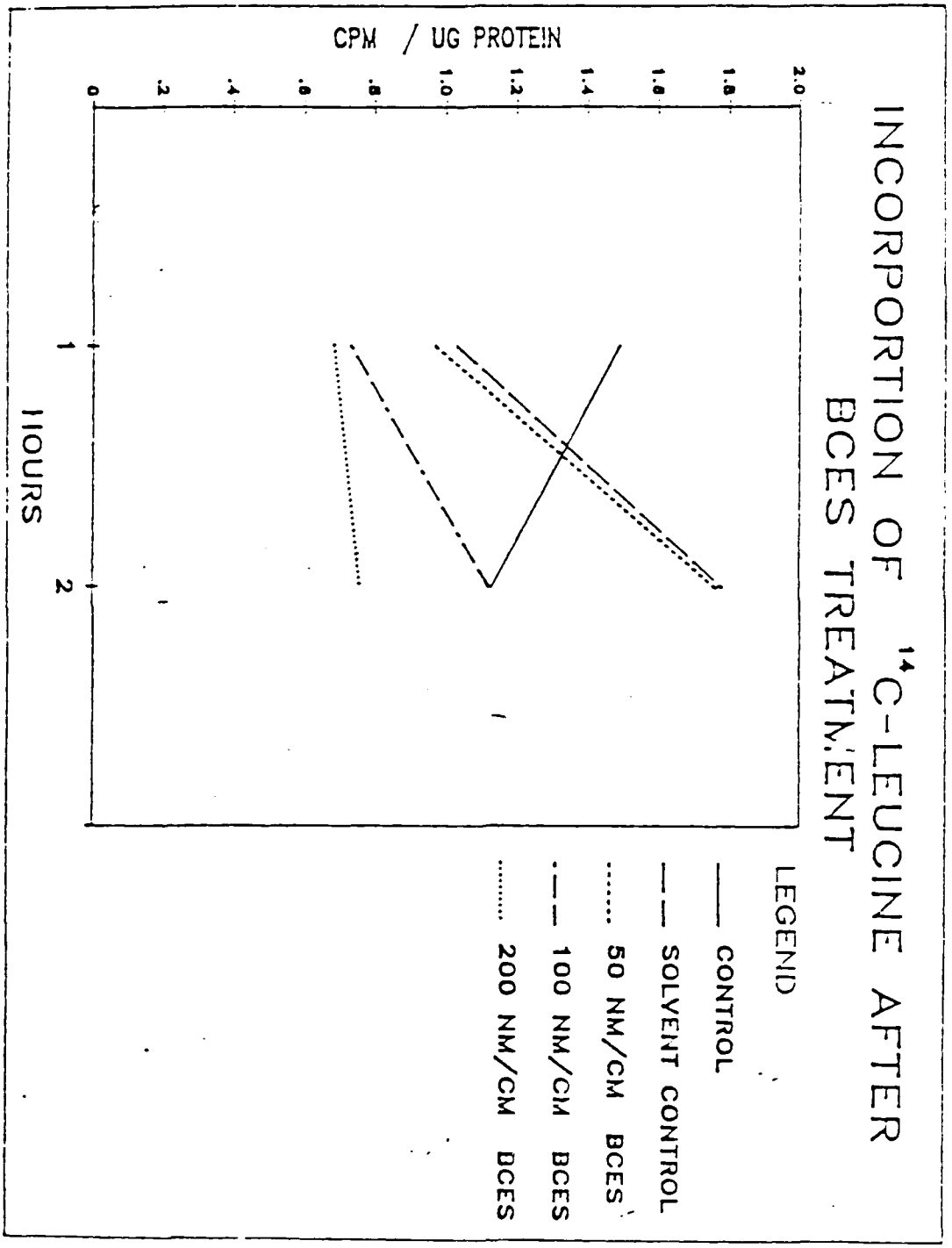
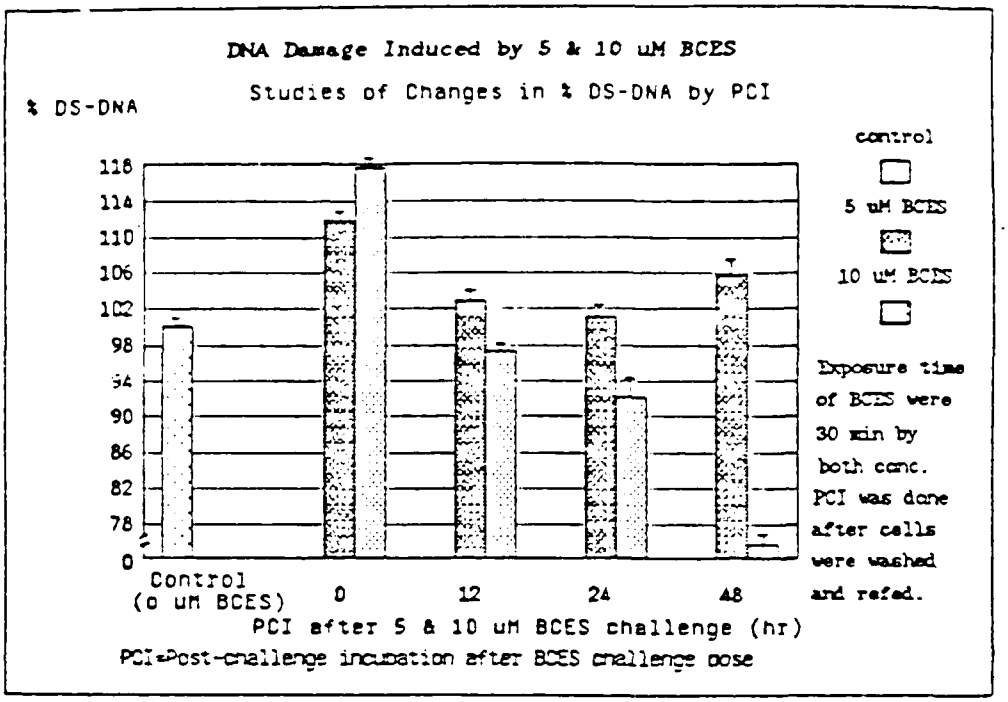

INCORPORTION OF ^{14}C-LEUCINE AFTER BCES TREATMENT
CPM / UG PROTEIN
0
.2
.4
.6
.8
1.0
1.2
1.4
1.6
1.8
2.0
1
2
HOURS
LEGEND
CONTROL
SOLVENT CONTROL
50 NM/CM BCES
100 NM/CM BCES
200 NM/CM BCES

Figure 4



Materials: Nine day old keratinocytes cultures grown in normal Ca^{++} (1.8 mM) were used in the experiments. Basal cells were isolated by the methods of pertol gradients and seeded in 35 mm plastic petri dishes at the density of 0.2 % (approximately a half million cells per ml). Cells were every other day with fresh complete medium containing 10 % fetal calf serum, insulin, and antibiotics.

Methods: To see DNA damage induced by 5 and 10 uM BCES challenge, cultures were exposed to both concentrations for 30 min. Post-challenge incubation (PCI) was carried out to see the changes in % DS-DNA (PCI time was 12, 24 and 48 hr). Quantification of DNA damage was carried out using alkaline unwinding assay (AUA), hydroxylapatite chromatography and fluorometric DNA analysis. AUA was done for 1 hr at room temperature in the dark using lysis solution which has a high pH (12.8).

Exposure: Cultures were 9 day old at the time of exposure to 5 and 10 uM BCES. Exposure time was 30 min for both concentrations. PCI was carried out to see the changes in % DS-DNA over time. At each time point by PCI (12, 24 and 48 hours), % DS-DNA was measured by the assays described in methods section.

Graph: Normalizing control DS-DNA as 100%, increase in % DS-DNA after 5 uM or 10 uM BCES challenge indicate cross-linking of DNA. Time course of PCI shows changes in % DS-DNA.
Error bars indicate S.E. (standard error) of experiments with sample size n=4 for control group and with n=6 for BCES exposed groups from two separate experiments. In any cases, S.E. < 5%.

TABLE 2

Protection against BCES-mediated damage by prior exposure to non-toxic level of MNNG

BCES challenge (20 uM)	N	Duration of post-challenge incubation (hr)	Pretreatment control (vehicle only) % control double-stranded DNA	MNNG Pretreatment % control double-stranded DNA
No	3	-	100 (a)	100 (b)
Yes	3	0	118 ± 4	113 ± 5
"	3	2	76 ± 2	106 ± 5
"	2	6	96	109
"	3	24	78 ± 5	65 ± 2

(a) 58 ± 6 of DNA double stranded.
(b) 49 ± 3 of DNA double stranded

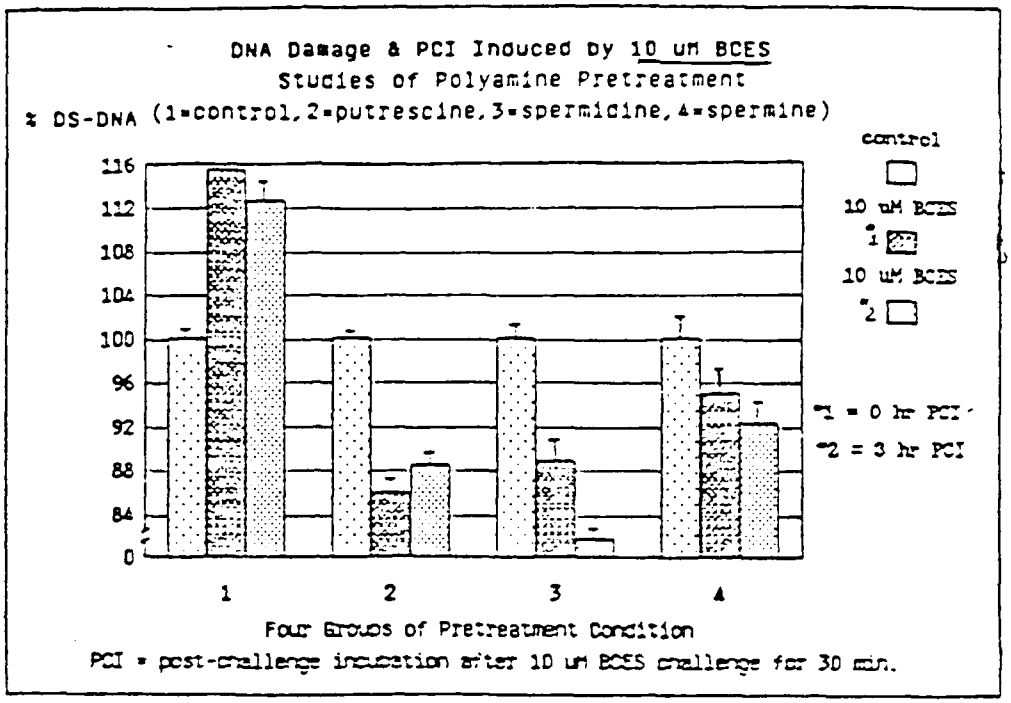
d. Effect of BCES on mitochondrial integrity

BCES could interfere with mitochondrial function (cf Section II, above). Inhibition of oxidative phosphorylation would be expected to initiate a sequence of reactions detrimental to the function and survival of the keratinocyte.

The rate of lactate production by cultured cells may increase when oxidative metabolism is inhibited (Penninck and Seinen, 1980; Brabec, et al., 1984; Miller, et al., 1985). Lactate accumulates at an increased rate because the normal route of lactate oxidation via oxidation of pyruvate and the Tricarboxylic Acid Cycle, has been blocked. The rate of lactate production may also be stimulated as the rate of glycolysis is increased to supplement the production of ATP by extra-mitochondrial routes. Therefore, production of lactate by cultured keratinocytes could be a method of indirectly determining whether exposure to BCES results in an inhibition of mitochondrial function in these cells.

Confluent 6-day old cultures produced lactate in a linear manner for at least 10 hours (Figure 6). Dinitrophenol (50 uM) stimulated the rate of lactate accumulation. Addition of carrier solvent (ethanol:methylene chloride, 4:1) to the medium also stimulated the rate of lactate

Figure 5



Materials: Basal cells isolated by "percoll gradients" were plated in 35 mm plastic dishes at the density of 2.5×10^5 per ml and fed with minimum essential medium (MEM) containing 10% fetal calf serum supplemented with insulin and antibiotics. These cultures were divided into four groups for pretreatment condition.

Group 1. - control, no pretreatment
Group 2. - pretreatment with putrescine 20 uM (4 day old to 8 day old)
Group 3. - pretreatment with spermidine 10 uM (4 day old to 8 day old)
Group 4. - pretreatment with spermine 10 uM (4 day old to 8 day old)

Methods: Quantification of DNA damage was carried out using alkaline unwinding assay (AUA), hydroxylapatite chromatography, and fluorometric DNA analysis.

Exposure: Nine day old keratinocytes cultures were used for BCES exposure. BCES challenge dose was 10 uM for 30 min. After the challenge dose, cells were washed once with DPBS (pH 7.4) and refed with fresh complete medium (37° C). Post-challenge incubation (PCI) was carried out for each group for 3 hours.

Graph: In each pretreatment condition, control samples were normalized as 100%. Percentage DS-DNA more than 100% indicate cross-linking of DNA by 10 uM BCES challenge. Three hours PCI was carried out to see the changes in DS-DNA (%) in each pretreated condition.
Error bars indicate S. E. with sample size n=6 for control, with n=6 for BCES exposed cells from two separate experiments. In any cases S. E. < 5 %.

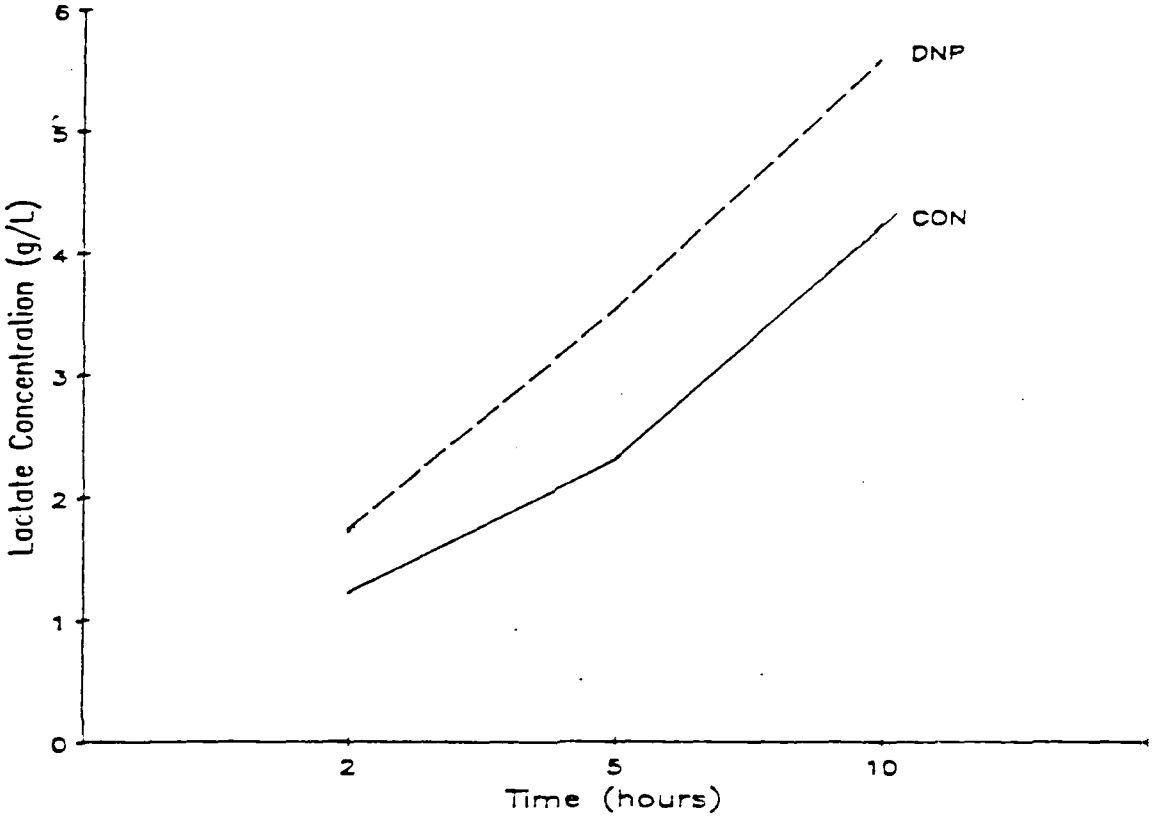


FIGURE 6

Lactate production by 9-day old cultures of rat keratinocytes was stimulated by 2,4-dinitrophenol (DNP), an uncoupler of oxidative phosphorylation. Basal cells were plated in 35 mm dishes and exposed to 50 uM DNP for 10 hr. Each time point represents an average of a duplicate determination on each of three samples. CON = Control.

accumulation. Exposure of cultures for 15 min to BCES at concentrations between 100 and 300 uM inhibited rates of lactate accumulation (Figure 7). BCES at concentrations below 100 uM did not significantly inhibit lactate production. This suggests either that the rationale for these experiments was incorrect or that mitochondrial function is not affected by exposure of keratinocytes to a concentration of BCES which strongly affects the integrity of DNA.

In a further effort to determine a metabolic effect of exposure to a low level of BCES on keratinocytes in culture, protein synthesis was studied. The technique involved the incubation of proliferating cultures with [^{3}H]leucine (10 uCi/plate;180 Ci/mmole) or [^{14}C]leucine (5 uCi/plate; 30 uCi/mmole) for 30 min followed by the addition of 10% trichloroacetic acid to stop the reaction and precipitate the protein which was collected on filters and processed for determination of radioactivity (Brabec, et al., 1974). BCES at concentrations up to 100 uM had no significant effect on the incorporation of radioactive leucine in the cultures (Table 3).

The utilization of glucose was also studied. Cultures of keratinocytes utilized glucose at a rate of 0.2 - 0.4 mmole/hour. Rates of utilization were stimulated by trifluormethoxy-carbonyl cyanide phenylhydrazone (FCCP) and the methylene chloride:ethanol carrier solvent. BCES also appeared to increase glucose consumption (Table 4).

It is, of course, possible that a metabolic abnormality may be a secondary contributor to the cellular necrosis associated with exposure to BCES, in vivo. The reality of this possibility may well be defined in later experiments of this project when, as contemplated in the present proposal, experiments will be done to determine whether the lesion in DNA caused by exposure to a low concentration of BCES, is sufficient to cause cellular necrosis in the culture.

Because mitochondrial function may be inhibited as a secondary effect of BCES exposure, it was necessary to determine if mitochondrial function could be directly inhibited. Therefore the effect of BCES on the repiration of mitochondria from rat liver was determined. Rat liver was used rather than epidermis because of the availability of larger amounts of material from the liver. As far as is known oxidative phosphorylation is comparable in the two tissues.

Concentrations of BCES less than 250 uM did not inhibit the rate of respiration of mitochondria from rat liver with succinate and glutamate as substrates. The solvent (methylene chloride/ethanol) strongly uncoupled mitochondria and prevented respiration in assays of concentrations of BCES greater than 250 uM. Since the concentration of BCES must be greater than 250 uM to inhibit respiration, it seems unlikely that mitochondrial dysfunction is a major concomitant of early toxicity from this chemical.

The action of BCES on the integrity of mitochondrial membranes in situ was examined using the localization of Rhodamine 123 as the assay. This substance binds to energized mitochondrial membranes (Johnson, et al,

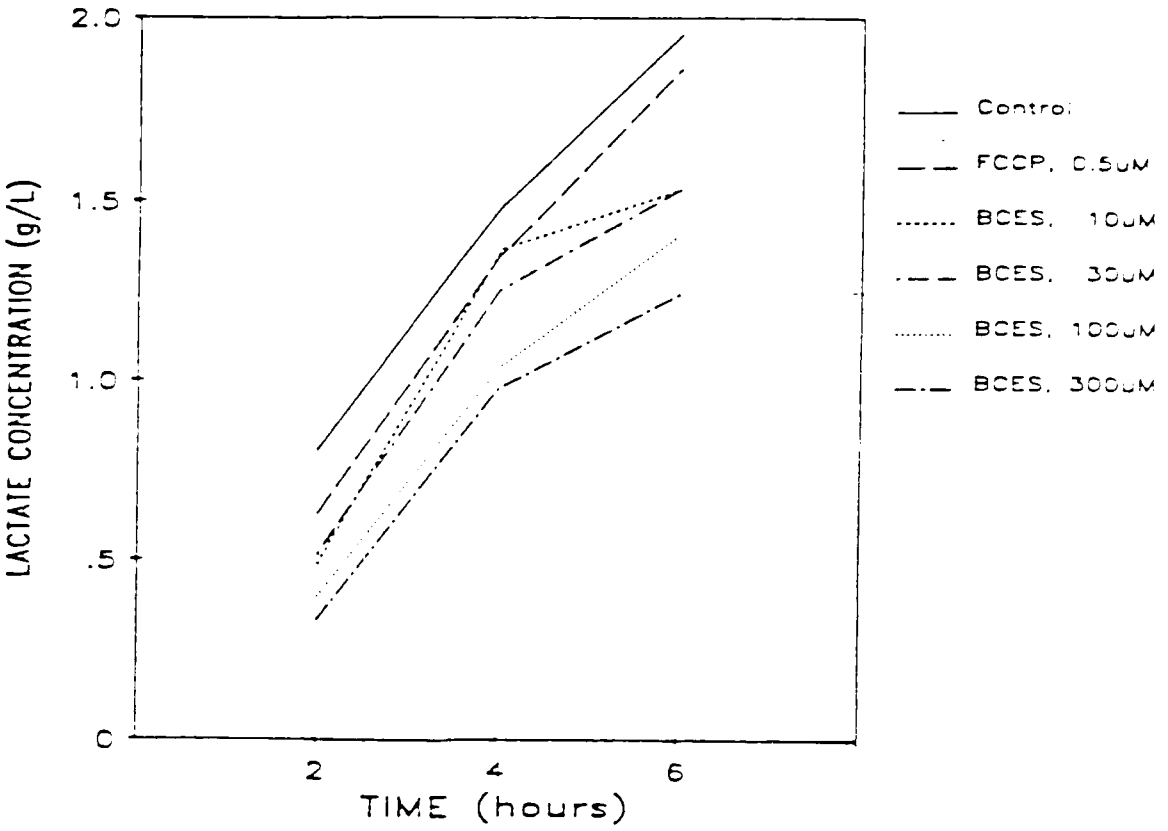


FIGURE 7. Lactate production by rat keratinocyte cultures was inhibited by exposure to BCES for 10 min. In this typical experiment, 3 dishes of cells were used for each exposure and duplicate samples were withdrawn for determination of lactate.

1980) and the mitochondrial profiles can be visualized by epifluorescence at appropriate wave length and high magnification in the light microscope. The dye diffuses from the mitochondria when the membrane is deenergized, e.g. when the mitochondria are uncoupled (Johnson, et al.,1982).

Preliminary results have been obtained with Rhodamine 123 staining of basal cell cultures. Cultures have not been exposed to BCES at this time. The Rhodamine-stained basal cells display a bright area of staining surrounding the nucleus that at high magnification appears as fine, dense filagree of fibers that become more diffuse toward the margin of the cell. All cells do not appear to be uniformly brightly stained, although all cells do stain. The degree of staining may be a result of differentiation that is presumed to be different for each cell in the 9 day-old basal submerged culture maintained in the low calcium medium. Application of an uncoupler (e.g. 0.5 uM p-trifluoromethoxy phenylhydrozone) to the culture causes the flourescence to lose its localized pattern and to become more diffuse throughout the cytoplasm. A diminution of total fluorescence may also be present although we cannot calibrate the quantum yield of the dye with present instrumentation and software.

TABLE 3

Effect of Low Concentrations of BCES on Protein Synthesis.

Treatment	CPM/Plate	% Control
Control	1323 ± 388	100
uM BCES		
10	800 ± 377	64
30	1507 ± 535	114
100	1001 ± 121	76
300	254, 260 (n=2)	20

Cultures of basal cells were exposed to BCES dissolved in EBBS for 15 minutes. The medium was withdrawn and replaced with fresh medium. After six hours, the cultures were rinsed and the medium replaced with EBBS containing 4.0 uCi [^{3}H]leucine per ml. After 30 minutes, the acid-precipitable radioactivity was determined. Values above are averages of 3 experiments, 3 samples, per experiment.

TABLE 4

Effect of Low Concentrations of BCES on the Rate of Glucose Consumption

Sample	Glucose Consumption, umole/L/hr
Control	0.047
uM BCES	
20	0.127
50	0.169
100	0.255
300	0.231

e. Development of techniques to study metabolism of BCES

The activities of two enzymes which could play a role in the metabolism of BCES have been examined in subcellular fractions prepared from whole skin, dermis and epidermis of 4-day old rats. The strain of rat was the same as used for the cultivation of keratinocytes. The results of a study in vivo by Davison, et al (1961) suggested that the initial glutathione-BCES adduct undergoes further degradation to generate different metabolites. Using optimal assay conditions, the presence of several enzymes was established (Table 5).

Glutathione S-transferase (GSHTr) was assayed using 1-chloro-2,4-dinitrobenzene as a model substrate (Habig, et al.,1974). Separation of dermis from epidermis was achieved by a modification of the method of Epstein, et al. (1979). Measurable GSHTr activity was found in both the dermis and the epidermis (Table 6) with the largest percentage of the enzyme in the dermis. Using a new rapid HPLC method in combination with affinity chromatoraphy substantial purification of this enzyme was achieved from the whole skin (Table 7).

TABLE 5

The activities of some marker and xenobiotic metabolizing enzymes in subcellular fractions of cutaneous cells

Enzyme/fraction	Enzyme activity nmoles/min/mg protein
Lactic dehydrogenase (cytosol)	1390
Alcohol dehydrogenase (cytosol) with NAD+	3.3
Acetaldehyde dehydrogenase (mitochondria) with NADP+	3.6
with NAD+	7.2
Acetaldehyde dehydrogenase (microsomes) with NADP+	23.0
with NAD+	12.0

An increase in absorbance at 340 nm resulting from the production of NADH or NADPH was monitored. Lactae, ethanol and acetaldehyde were used as the substrates. A millimolar extinction of 6.22 for the reduced form of the pyridine nucleotide was used to calculate enzyme activity. Tissue was fractionated by homogenization in 0.05M Tris, pH 7.4, and submitted to differential centrifugation to yield the different fractions.

TABLE 6

Distribution of cytosolic glutathione S-transferase (GSHTr) activity in neonatal rat skin

Fraction	Volume (ml)	Protein (mg/ml)	Total (mg)	GSHTr Activity Specific Activity (nmol/min/mg)	Total Activity (nmol/fraction	% Distribution
Whole Skin	32	0.098	35.14	48	1690	100
Dermis	48	1.028	49.34	47	2320	88
Epidermis	18	0.576	10.37	31	310	12

3-day old rats (n=10-20 each) were used. GSHTr activity was assayed according to Habig, et al. (1974), using CDNB as a substrate. The data represent the results of a typical experiment. Similar results were obtained in 2 additional experiments.

TABLE 7

Purification of glutathione S-transferase (GSHTr) from whole skin of 3 day old rats.*

Fraction	Specific Activity (mol/min/mg)	Fold Purification	% Recovery
Crude Cytosol	0.034 ± 0.007	1	100
Affinity Chromatography	29.88 ± 7	1021 ± 367	106 ± 20
HPLC-Applied	12.5	1	100
-Recovered	35.0	2.8	122

*GSETr activity was assayed according to Habig, et al. (1974), using CDNB as a substrate. The data represent mean ± S.E. (n=3 to 7) or the results of a typical experiment. The cytosolic fraction (100,000 xg supernate) was submitted to affinity chromatography on GSH-Sepharose 4B. The active fraction was eluted with 10 mM GSH, pH 9.4, concentrated by ultrafiltration and further purified by HPLC using an anion exchange column.

Peroxidase was assayed using guaiacol (O-methoxy phenol) as a model substrate (Himmelhoch, et al.,1967). This enzyme was predominantly associated with the nuclear and mitochondrial fractions derived from whole skin (Table 8). An approximately 80-fold purification of this enzyme was achieved starting with a whole skin homogenate (Table 9).

TABLE 8

Subcellular distribution of the cutaneous peroxidase activity in 3-day old rats.*

Subcellular Fraction	Peroxidase Activity (Units/mg)	% Distribution
Cytosol	0.000	0.00
Nuclear	0.171	36.90
Mitochondria	0.263	57.18
Microsomes	0.025	5.92

*Peroxidase activity was measured according to Himmelhoch, et al. (1967) using guaiacol as a substrate. One enzyme unit is defined as the amount of enzyme that causes a change of 1.0 absorbance unit per min at 470 mm. The results are mean peroxidase activity observed in 2 separate experiments. Each enzyme preparation represents pooled material from 20-30 rats.

TABLE 9

Purification of neonatal rat skin peroxidase.*

Fraction	Specific Activity (units/mg protein)	Fold Purification	% Recovery
Crude Extract	0.29	1	100
Concavalin A	7.17	24.7	70
Bio Gel P-150	24.00	82.0	15

*The data represent the results of a typical experiment. The details of the purification procedures are given in the text. See Footnote to Table 9 for additional details.

E. Conclusions

1. Obtaining stratified terminally differentiating cultures

The use of attachment factors appears neither useful nor necessary. This effort will no longer be pursued.

The stratified cultures can be exposed to BCES dissolved in 70% DMSO. It appears that the BCES attacks the lower cells by passing through the tissue rather than around it into the medium but this has not been proven as yet.

2. Proliferating monolayers of keratinocytes

Growing keratinocytes in low calcium medium produces cultures which remain as monolayers although cells in the monolayer do exhibit early steps in differentiation. This culture is particularly useful for studying the direct interaction between BCES and the cell since the toxicant is added to the medium in which the cells are immersed.

3. Lifted cultures exhibit changes in cell surface carbohydrates consistent with those seen in situ.

4. It appears from preliminary data that BCES-mediated cross-links can be repaired but other more sensitive and definitive techniques will have to be applied to confirm this conclusion. Preliminary data also suggest that the pattern of DNA damage from BCES can be altered by inducing repair synthesis prior to BCES exposure and, possibly, by supplementation with polyamines. Again further work is necessary to verify this idea.

5. Metabolic effects such as alteration in mitochondrial respiration, utilization of glucose, protein synthesis and RNA synthesis require higher levels of exposure than isnecessary to affect the integrity and metabolisnm of DNA.

F. Recommendations

The biological systems developed in this project are appropriate for use in determining the molecular and cellular mechanisms responsible for the toxic manifestations associated with vesication. Attention should now be focused on determining the most sensitive indicator of toxicity in order to define the nature of the molecular interactions which lead to toxicity from BCES. In this context, the keratinocyt's ability to repair molecular and cellular damage done by BCES should be evaluated. The objective of further study should be to develop a profile of molecular and cellular alterations in relation to increasing levels of exposure to BCES. This kind of information should provide an understanding of the kinds of BCES-mediated effects which the cell can accommodate and identify those which exceed its ability to tolerate leading to overt toxicity.

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